

# Selection for Aluminum and Acid-Soil Resistance in White Clover

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## ABSTRACT

Despite good resistance to acid-soil stresses, white clover (*Trifolium repens* L.) is not found on some acid soils. Our objectives were to develop a large-leaved white clover with acid-soil resistance, to relate seedling Al to mature plant acid-soil resistance, and to validate our soil-on-agar procedure. We used a two-stage selection procedure. In Stage 1 we used the soil-on-agar technique to select for seedling Al-resistance and Al-susceptibility in Brown Loam Synthetic No. 2 and 'Grasslands Huia' white clover. In Stage 2 we used conventional pot studies with two soil pH treatments, 4.2 and 5.2, and stem-tip cuttings of Al-resistant selections from Brown Loam to select for acid-soil resistance. The same Stage 1 and 2 techniques were used to evaluate 12 experimental populations and both parents for seedling Al resistance and mature plant acid-soil resistance. Across two cycles of selection, both Brown Loam and Huia Al-resistant and susceptible populations diverged. For Brown Loam, progress was made toward both increased Al resistance and susceptibility. For Huia, progress appeared more toward Al susceptibility than toward Al resistance. Populations developed from two-stage selection were more acid-soil resistant than their parent. However, populations selected only for seedling Al resistance or Al susceptibility were usually no more acid-soil resistant than their parent. We were able to increase the acid-soil resistance of Brown Loam white clover. But, the soil-on-agar procedure was not an effective technique for developing acid-soil-resistant white clover germplasm.

FROM A PHYSIOLOGICAL PERSPECTIVE, a plant's response to stress, for example, temperature extremes or drought, can be characterized as either avoidance (the factor is excluded from the plant tissue) or tolerance (the factor penetrates the tissue but the tissue survives) (Levitt, 1964). Kochian (1995) extended this mechanistic terminology to stress from Al using the terms *exclusion*, rather than *avoidance*, and *tolerance*. Physiologically, the term *resistance* is mechanism neutral; that is, it implies neither tolerance nor exclusion. In this paper, we will use the term *resistance* to characterize the response of white clover to Al and acid-soil stress.

White clover is relatively resistant to acid-soil stresses, but it is not found on some highly acid soils even though it can be abundant on adjacent sites where pH is higher. On such low-pH soils, initial seedling establishment is especially critical, although vegetative persistence is also important, in determining white clover presence or absence (Voigt, 1997, unpublished data). Toxic levels of Al are believed to be a major component of much of this acid-soil stress.

The classical procedure used to determine affects of Al on plants is growth in solution culture. When soil-

based studies are conducted, the effects of Al are usually confounded with other factors such as levels of P, Ca, Mo, Mn, Zn, organic acids, and other soil components (Edmeades et al., 1995; Villagarcia et al., 2001). An exception is studies of the growth of very young seedlings. This is possible because (i) the effect of Al on root growth is rapid, occurring within hours, and can be dramatic (Kochian, 1995), (ii) Al and proton activity at the root surface are the major factors limiting white clover primary root growth, with Al being more toxic than protons (Brauer, 1998), and (iii) initial root growth is dependent primarily on nutrient reserves in the seed rather than nutrients from the soil (Edmeades et al., 1995).

The soil-on-agar technique (Voigt et al., 1997) takes advantage of the above factors to provide a biological assessment of soils at the critical establishment stage that is closely related to the toxic form of Al found in the soil solution (Voigt et al., 1998). The procedure can be used to relate seedling Al resistance to acid-soil resistance of small-seeded forage legumes (Voigt et al., 1997; Voigt and Mosjidis, 2002). For this paper, results from the soil-on-agar procedure will be discussed as Al resistance, results from pot studies as acid-soil resistance.

As a species, white clover occurs on a wide array of soils that range from calcareous to acid (Snaydon, 1962). Plants from populations growing on acid soils were better adapted to these soils than plants from calcareous soils (Snaydon and Bradshaw, 1962). Also, plants from the acid-soil populations were less well adapted to other soils or to high levels of lime application. Extensive variation in Al resistance among >50 white clover cultivars was reported by Wheeler and Dodd (1995). Solution Al activity, at which root yields were reduced by 50%, ranged from 0.7 for 'Menna' to 3.0 for 'Pronitro'. Similar variability was observed for herbage yields. However, in soil-based studies where  $\text{Al}(\text{SO}_4)_3$  was added to a pH 5.0 soil to induce stress, differences were not detected in acid-soil resistance among white clover cultivars (Caradus et al., 1987; Mackay et al., 1990). The experiment did suggest, however, that variation existed within white clover cultivars and that selection for acid-soil resistance within otherwise adapted germplasm might be a useful approach to white clover improvement.

Genotypes with increased acid-soil resistance and susceptibility were identified from Huia white clover (Caradus et al., 1991). Results from a  $6 \times 6$  diallel cross of three resistant and three susceptible genotypes suggested that acid-soil resistance was recessive. Distributions of acid-soil resistance among the hybrids illustrate the problem of breeding for this character (Caradus and Mackay, 1995). Regardless of parental classification as resistant or susceptible, most hybrids were susceptible. However, crosses between acid-soil-resistant genotypes

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**Abbreviations:** CoV<sub>E</sub>, coefficient of velocity of root emergence; T50E, time to 50% root emergence.

did produce more resistant hybrids (11%) than crosses between susceptible parents (3%). Crosses between susceptible parents did produce many more highly susceptible hybrids (30%) than crosses between resistant parents (9%). These findings favor the genetic approach of selecting resistant germplasm from within adapted populations rather than attempting to locate genes for resistance in unadapted germplasm and then needing to transfer a recessive characteristic from poorly-adapted to well-adapted cultivars. The results also suggest the likely need for more than one cycle of selection to achieve reasonable levels of acid-soil resistance in white clover populations.

Our objectives were to evaluate the effectiveness of the soil-on-agar procedure as a selection technique and to determine if populations selected for Al resistance and susceptibility as seedlings were also more acid-soil resistant and susceptible as older plants. We hypothesized that seedling Al resistance, as identified by the soil-on-agar technique and expressed in the primary root, would be related to mature-plant acid-soil resistance and could be used to increase efficiency of selection for acid-soil resistance. A final objective was to develop a large-leafed white clover population with improved acid-soil resistance. We used Brown Loam Synthetic No. 2 (Caradus and Woodfield, 1997) as a parent. Brown Loam is a large-leafed germplasm known to have the potential for high production and good persistence in the hill-land environment of Appalachia (Voigt and Morris, 1995, unpublished data). We used Huia, a medium-leafed, grazing-tolerant cultivar (Caradus and Woodfield, 1997), known to contain genetic variation for acid-soil resistance (Caradus et al., 1991), to further study the first two objectives.

## MATERIALS AND METHODS

### Seedling Aluminum Selection

Brown Loam (BL) and Huia (H) seedlings were screened for Al resistance with the soil-on-agar procedure. The soil used was a single lot of thoroughly mixed Porters soil (coarse-loamy, mixed, mesic Typic Dystrudepts), containing A and Bw horizons. Soil pH was close to the desired pH of 4.2 to 4.4 and adjustment of pH with  $\text{CaCO}_3$  was not needed. Table 1 lists the chemical analysis of this soil. The soil-on-agar procedure was previously described (Voigt et al., 1997). Briefly, a layer of moist soil  $\approx 8$  mm deep was gently and uniformly distributed on top of solidified agar ( $5 \text{ g L}^{-1}$  agar in distilled water) in a rectangular, clear, plastic flask. Germinated seeds, with a radical length of  $\approx 1$  mm, were planted immediately below the soil surface. Flasks containing 18 seedlings each were set on trays and placed in a growth chamber (12-h light at  $\approx 5 \mu\text{mol m}^{-2} \text{ s}^{-1}$  at  $23^\circ\text{C}$ , and 12-h darkness at  $15^\circ\text{C}$ ). Root emergence from the soil into the agar was observed daily for 10 d.

Two seedlings, one with the fastest and one with the slowest root emergence, were selected from each flask. In most flasks, roots of one to as many as three seedlings did not grow. Root tips of these seedlings were somewhat enlarged and had a stubby appearance typical of Al-injured roots (Teraoka et al., 2002). If, at the end of the trial, a healthy seedling with a stubby root was discovered, it was selected in place of the seedling with the slowest root emergence. When more than

**Table 1. Chemical analysis of Porters soil used in selection and from small pot evaluation experiments.**

Experiment	pH <sub>w</sub>		Mn†	Mg†	Ca†	K†	P†	Al†	Al saturation
	Initial	Final							
	mg kg <sup>-1</sup>								%
Selection‡	4.2	—	—	22	135	86	4	225	70
	5.0	—	—	19	790	78	3	64	14
	5.3	—	—	17	1150	79	3	19	3
Small-pot evaluation§	4.2	4.2	13	13	358	40	146	167	48
	4.7	4.6	13	12	912	44	149	76	15
	5.6	5.1	6	9	1546	44	134	18	2

† Chemical methods: ammonium acetate extraction, Bray phosphorus, and potassium chloride extraction Al.

‡ Soil representative of that used in all selection experiments without amendments except 0, 2, or  $3 \text{ g kg}^{-1} \text{ CaCO}_3$ .

§ Soil from pots following completion of the small-pot evaluation experiment.

one otherwise healthy seedling with a stubby root occurred, one of them was selected at random. Seedlings were transplanted into a commercial potting mix and grown in a greenhouse. Seedlings from 110 flasks with slow and fast emerging roots were saved for the Al-susceptible (BL-AIS-C1) and resistant (BL-AIR-C1) populations, respectively. Similarly, seedlings from 60 flasks of Huia were saved for H-AIS-C1 and H-AIR-C1 populations.

A second cycle of selection was completed by selecting a slow emerging seedling from each of 125 flasks of the BL-AIS-C1 population to produce a BL-AIS-C2 population. Similarly, 125 fast emerging seedlings were selected from BL-AIR-C1 to produce a BL-AIR-C2 population. A second cycle of selection was not undertaken with the Huia populations.

### Acid-Soil Resistance Selection

Brown Loam was subjected to a tandem selection procedure. The first stage of selection used the soil-on-agar procedure as described above. Three trials of 110 flasks each were completed, resulting in selection of 330 seedlings with slow and fast emerging primary roots, Al-susceptible and Al-resistant, respectively.

About 12 wk following transplanting, plants from all 330 Al-susceptible selections were visually selected for plant vigor by two observers. The purpose of this selection was to counter possible negative effects of selecting for slow root growth on plant vigor. The 37 most vigorous plants, from each run of 110 plants (33%), were selected and saved for seed production. A total of 110 plants were saved to form an Al-susceptible population, BL-AIS<sub>33</sub>-C1. This population was included to provide a population that would have the same number of plants as, and that could be compared with, the acid-soil-resistant populations described below. We did not create acid-soil susceptible populations because of the extensive time and effort that would have been required to develop them from the Al-susceptible selections.

Seedlings of all Al-resistant selections were grown until they produced several stolons. Six stolon tips were cut from each selection and were rooted in potting mix. The four most vigorous cuttings from each selection, as determined after removing the cuttings from the mix, were used in small pot studies to determine acid-soil resistance.

Three studies, composed of 110 Al-resistant selections each, were conducted. Each study used Porters soil at two pH levels,  $\approx 4.2$  and adjusted to  $\approx 5.2$  with  $\text{CaCO}_3$ , and two replications. With the addition of  $\text{CaCO}_3$ , the Al saturation of the soil decreased by  $\approx 60\%$  (Table 1). Before final pH adjustment, nutrients were added at 75, 100, and  $125 \text{ mg kg}^{-1}$  soil of N, P, and K, respectively, along with micronutrients, sufficient to ensure good plant growth. Because observations from a field

study, conducted at pH levels similar to those used for selection, indicated an absence of effective nodulation of white clover (Voigt and Morris, 1996, unpublished data), plants were grown symbiotically. Studies were conducted in a growth chamber with environmental conditions conducive to good clover growth. Each pot, containing 482 g of Porters soil (at  $\approx 0.033$  MPa soil moisture) and 4 g of perlite to retard evaporation, weighed 500 g. Distilled water was added three times each week to bring each pot back to the 500-g weight. Each study was run for  $\approx 35$  d, at the end of which plants were washed from the soil, divided into roots and tops, dried, and weighed. Weights were adjusted for initial stolon tip weight by analysis of covariance. Resistance index ( $I_R$ ) for root weight (RW) was calculated on a within-replication basis, where:

$$I_R = (\text{RW at pH 4.2})/(\text{RW at pH 5.2})100.$$

Plants with the highest resistance index for root weight were selected; however, the mean root weight of all selected plants in the pH 5.2 soil also had to be approximately equal to that of all 110 plants evaluated. Thus, plants with below-average root weight in the pH 5.2 soil could only be selected if their root weights were counterbalanced by other plants whose root weights were above average. The intent was to select plants with increased acid-soil resistance while avoiding changes in plant vigor in higher pH soils. In Cycle 1, three intensities of selection were used (33, 17, and 9%), resulting in selection of 37, 19, and 10 plants from each run, or a total of 111, 57, and 30 plants from all three runs. Thus, three acid-soil-resistant populations (BL-ASR<sub>33</sub>-C1, BL-ASR<sub>17</sub>-C1, and BL-ASR<sub>9</sub>-C1) were created.

A second cycle of selection was conducted with the BL-ASR<sub>17</sub>-C1 population. This population was chosen because we were concerned that the selection intensity of the BL-ASR<sub>33</sub>-C1 might have been too low and that the BL-ASR<sub>9</sub>-C1 population might be subject to inbreeding depression. Procedures were essentially as described above for Cycle 1 except that the second stage was conducted in a greenhouse rather than in a growth chamber and fewer plants were evaluated and selected than in the first cycle of selection. About 240 Al-resistant plants were selected from the first stage soil-on-agar runs and were evaluated for acid-soil resistance in the second stage of selection. Only 24 plants, 10% of those evaluated, were selected to form the second cycle acid-soil-resistant population, BL-ASR<sub>10</sub>-C2.

In addition, the acid-soil-resistant Huia population H-ASR-C1 was developed from the 50 most vigorous plants recovered from a field seeding,  $\approx 84$  m<sup>2</sup> in size seeded at  $\approx 600$  seed m<sup>-2</sup> of Huia. The soil was a Gilpin silt loam (fine-loamy, mixed, active, mesic, Typic Hapludults) with a pH of  $\approx 4.9$  (Staley, 1999, personal communication).

### Seed Production

Selected plants were maintained during winter in a shaded greenhouse at cold but above-freezing temperatures (heat setting of 3°C) and induced to flower during late winter to spring by transfer to a greenhouse with warmer temperature and extended daylengths. Plants were pollinated in isolation cages by honey bees. Following pollination, seed was matured in a greenhouse, harvested, dried, and cleaned on an individual plant basis. Approximately equal quantities of uniformly cleaned seed from each plant were mixed to form each population. Plants that produced little or no seed were excluded from the populations. Conditions for seed production were better in populations with smaller numbers of plants. All plants selected for acid-soil populations (mean of 55 plants per population) were included. In the larger Al populations, a mean of

99 plants per population, 82% of the plants were included. Crowding of plants in the isolation cages was probably responsible for the poorer seed set of the largest populations.

## Evaluation of Progress from Selection

### Aluminum Resistance of Experimental Populations

All 12 selected and two base populations were evaluated with the soil-on-agar procedure to assess response to Al stress. Techniques were identical to those used in the selection experiments. Although Porters soil was used for the evaluation experiments, this soil was lower in pH than the Porters soil used during the earlier selection experiments. Even the soil pH of the most-acid treatments had to be adjusted upward with CaCO<sub>3</sub> to achieve similar pH levels. This changed not only the pH, but also the Al saturation levels which decreased from  $\approx 70\%$  at pH 4.2 in the original soil to  $\approx 50\%$  in soil with a pH of 4.2 used in these experiments (Table 1). Thus, despite the similar pH, levels of Al stress were lower.

Because only a limited number of flasks, the experimental unit, could be included in an experiment, several soil-on-agar experiments were run. In Exp. 1, Brown Loam and the BL-AIS-C1, BL-AIR-C1, BL-AIS-C2, and BL-AIR-C2 populations were compared at soil pH levels of 4.18, 4.38, 4.63, and 5.28. In Exp. 2, Huia and the H-AIS-C1, H-AIR-C1, and H-ASR-C1 populations were compared at soil pH levels of 4.18, 4.32, 4.60, and 5.23. In Exp. 3, Brown Loam and the BL-ASR<sub>33</sub>-C1, BL-ASR<sub>17</sub>-C1, BL-ASR<sub>9</sub>-C1, BL-ASR<sub>10</sub>-C2, and BL-AIS<sub>33</sub>-C1 populations were compared at soil pH levels of 4.39, 4.64, and 5.23. An additional experiment including a lower pH level did not include all the Exp. 3 populations and will not be presented in detail. All experiments contained five replications in a randomized block design.

Root emergence counts were made for 9 d. Initial observations, during periods of rapid root emergence, were made at 8-h intervals. As root emergence rates slowed, the interval between observations was increased to 12 and then to 24 h. At the end of an experiment, potential emergence was characterized as total number of seedlings, 18, reduced by the number of obviously defective seedlings and by the occasional seedling whose root growth, rather than proceeding into the soil, forced the seed up above the top of the soil surface by more than  $\approx 5$  mm. Root emergence counts were converted to a percentage of the potential emergence for each flask. Mean cumulative root emergence percentage (emergence) through 188 h was then calculated and analyzed.

To further characterize the root emergence curves for each experiment, Kotowski's coefficient of velocity (CoV<sub>E</sub>) through 188 h (Scott et al., 1984) and time to reach 50% of potential root emergence (T50E) were also determined as estimates of rate of root emergence. The CoV<sub>E</sub> was calculated as

$$\text{CoV}_E = 100(\Sigma N_i / \Sigma N_i T_i),$$

where  $N_i$  was the number of roots emerged at time  $i$  and  $T_i$  was the number of hours from planting.

Resistance index, in these experiments an assessment of Al resistance, was calculated as

$$I_R = (X \text{ at pH 4.2})/(X \text{ at pH 5.2})100,$$

where  $X$  was either emergence, CoV<sub>E</sub>, or T50E.

Data were analyzed with Proc Mixed (SAS Institute, 1997). Replications were considered random while pH treatments and entries were considered fixed. Differences between residual log likelihood estimates were compared with the  $\chi^2$  distribution to determine when separate variance groups were required for different pH treatments. Contrast statements and



*t* tests of least square means were used to make comparisons within cycles of selection and between populations and their predecessors and to examine population  $\times$  pH interactions.

### Acid-Soil Resistance of Experimental Populations

All selected and base populations were evaluated in a small pot study similar to those used for selection. Porters soil was adjusted to pH levels of 4.2, 4.7, and 5.6 with  $\text{CaCO}_3$ . Nutrients were added at 45, 60, and 75  $\text{mg kg}^{-1}$  soil of N, P, and K, respectively, along with micronutrients, sufficient to ensure good plant growth. Because an absence of effective nodulation at pH 4.2 was expected (Voigt and Morris, 1996, unpublished data), all plants were grown asymbiotically.

Seed were germinated and grown in a greenhouse in commercial potting mix to the third trifoliate leaf stage. Three seedlings were then transplanted into each pot and 4 g of perlite was added to each pot to retard evaporation. Pots were placed in a greenhouse in a randomized complete block design of eight replications. The experiment was conducted between 4 Dec. 2001 and 19 Feb. 2002. Mean night and day temperatures during that period were 18 and 22°C, respectively. Absolute minimum and maximum temperatures ranged from 9 to 31°C, but the mean daily absolute minimum and maximum were only 17 and 27°C, respectively. Lights were on for 11 h each day and provided a minimum photosynthetic photon flux density of  $\approx 200 \mu\text{mol m}^{-2} \text{s}^{-1}$  on cloudy days and during early morning and evening hours. Distilled water was added three times each week to bring each pot back to its original weight.

Leaf counts were made during Weeks 1, 4, and 9 of the experiment. At the end of the study ( $\approx 10$  wk after transplanting), plants were washed from the soil, divided into three components (roots, leaves and petioles, and primary stem base and any stolons) dried, and weighed. Data were analyzed with Proc Mixed (SAS Institute, 1997) as described above, with resistance index used as an assessment of acid-soil resistance. Although this experiment was conducted as a unified study, results will be presented in the same order and combinations of entries as in the three Al-resistance experiments.

At the conclusion of the experiment, soil from additional pots containing only two seedlings but watered identically to the experimental units was analyzed (Table 1). The pH of the most-acid treatment had not changed during the experiment but that of the highest-pH treatment had declined by 0.5 units. However, even at this pH the Al saturation was only  $\approx 2\%$  compared with 48% for the most-acid treatment.

## RESULTS AND DISCUSSION

### Aluminum Resistance of Brown Loam Aluminum Populations

At pH 5.28, primary root emergence from soil into agar of Brown Loam and all selected populations was relatively rapid (Fig. 1a). Emergence of the BL-AIR-C2 population was slower than that of other populations. That of the BL-AIS-C2 population, while not initially different from that of Brown Loam, reached almost 90% emergence at 68 h, almost 40 h before any other population achieved that level of emergence.

At pH 4.18, emergence was delayed. Emergence of the BL-AIS-C2 population continued to exceed those of other populations except at  $\approx 68$  to 92 h, when the BL-AIR-C1 population was similar or higher in emergence. In contrast to results at pH 5.28, emergence of

the BL-AIS-C1 population was noticeably slower than that of other populations.

Root emergence curves were quantified by examining emergence and  $\text{CoV}_E$  (Fig. 2a). Results were similar for both characters. At pH 5.28, the BL-AIR-C2 and BL-AIS-C2 populations differed, with the Al-susceptible population having greater emergence and  $\text{CoV}_E$  than the Al-resistant population ( $P < 0.05$ ). At pH 4.18, those differences were eliminated. In contrast, the BL-AIR-C1 and BL-AIS-C1 populations did not differ at pH 5.28 but did differ at 4.18 ( $P < 0.05$ ), at least for emergence. Because of the differences at pH 5.28, only the resistance index comparisons provide an assessment of Al resistance and susceptibility of the selected populations and the parent. Results for both characters indicated that we were successful in altering the seedling Al resistance and/or susceptibility of Brown Loam. Results for  $\text{CoV}_E$  indicated that divergence was not obtained until the second cycle of selection, while that for emergence suggested that divergence was obtained following one cycle of selection and was only maintained by the second cycle of selection ( $P < 0.05$ ). Results for  $\text{CoV}_E$  indicated also a relatively symmetric response to selection but that the base population was more similar to the BL-AIS-C2 than to the BL-AIR-C2 population. In contrast, results for emergence indicate that the base population was more similar to the BL-AIR-C1 than to the BL-AIS-C1 population. Considering both characters, it appears reasonable to conclude that the base population was intermediate in Al resistance and that progress from selection was achieved in both positive (resistance) and negative (susceptible) directions.

### Aluminum Resistance of Huia Populations

At pH 5.28 (Fig. 1b) Huia emerged more quickly and completely than any of the populations selected from it. Root emergence of both the H-AIR-C1 and H-AIS-C1 populations were clearly inferior to that of Huia. This difference was probably a reflection of the high seed quality of the commercial Huia seed. The Huia seed had a greater hundred-seed weight than any other seed lot included in these studies (data not presented) and it germinated more rapidly and uniformly than any experimental population or Brown Loam.

Huia emerged also more completely than other populations at pH 4.18, though its initial emergence through 80 h was not much different from that of the H-AIR-C1 or H-ASR-C1 populations. However, the most striking difference, compared with the higher pH, was the very slow and incomplete emergence of the H-AIS-C1 population.

The superiority of the Huia parent compared with the H-AIS-C1 and to a lesser extent the H-AIR-C1 populations are reflected in the emergence and  $\text{CoV}_E$  responses (Fig. 2b). For  $\text{CoV}_E$ , the H-AIS-C1 population was inferior to Huia and the H-ASR-C1 population at both pH levels ( $P < 0.05$ ). The H-AIS-C1 population was inferior to the H-AIR-C1 population only at pH 4.18 ( $P < 0.05$ ). For emergence, both the H-AIS-C1 and H-AIR-C1 populations were lower in emergence than

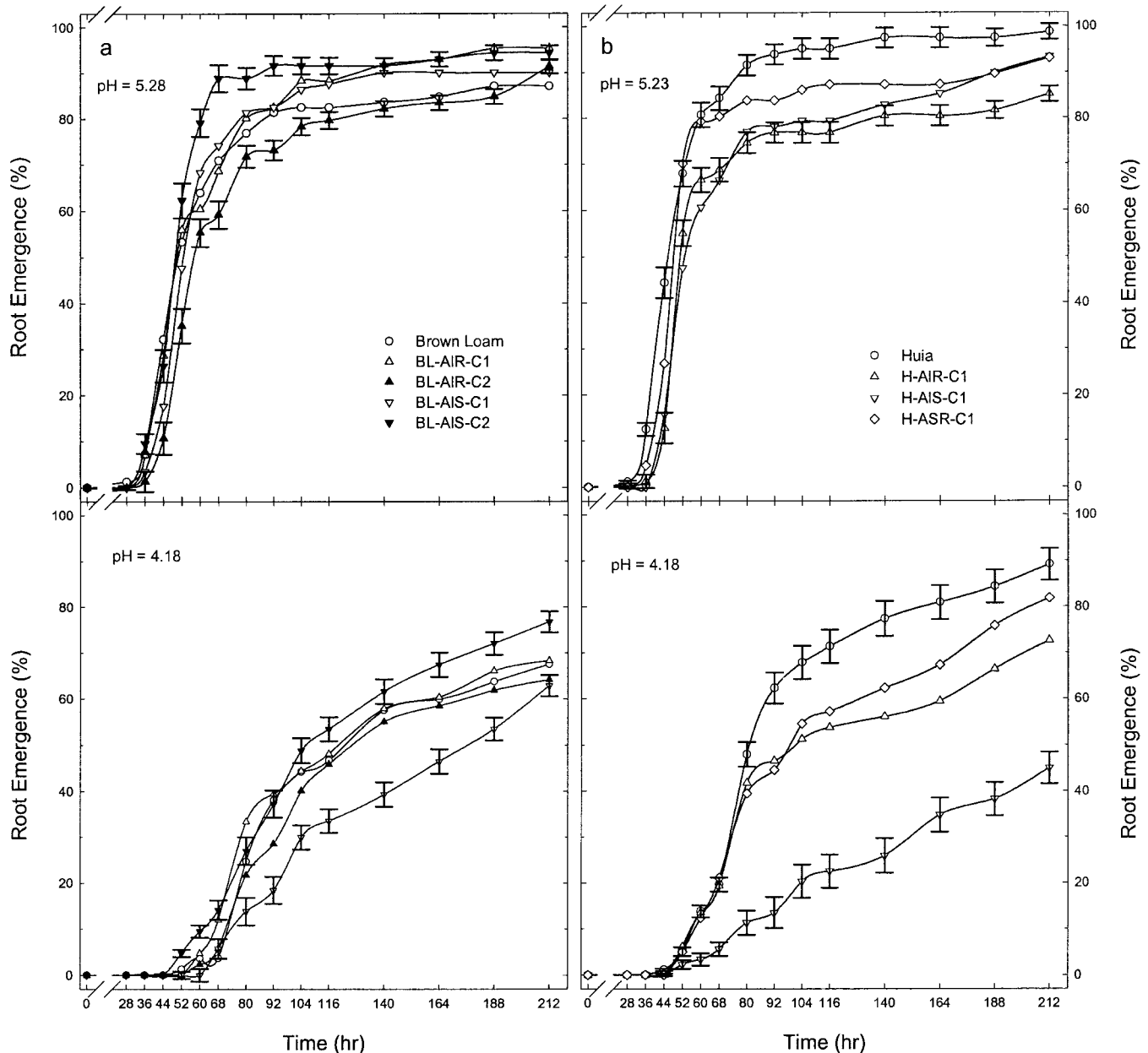


Fig. 1. (a) Effect of soil pH on root emergence across time from soil into agar of Brown Loam (BL) and the Al-resistant Cycles 1 and 2 (BL-AIR-C1, BL-AIR-C2) and susceptible Cycles 1 and 2 (BL-AIS-C1, BL-AIS-C2) white clover populations derived from it. (b) Effect of soil pH on root emergence across time from soil into agar of Huia (H) and the Al-resistant Cycle 1 (H-AIR-C1), susceptible Cycle 1 (H-AIS-C1), and the acid-soil-resistant Cycle 1 (H-ASR-C1) white clover populations derived from it. Standard error bars, shown for two populations per graph, apply to all populations.

Huia at both pH levels ( $P < 0.05$ ). Results for resistance index indicate, for either characteristic, that the H-AIR-C1 and H-AIS-C1 populations had diverged in Al resistance ( $P < 0.05$ ). However, the relationship of their performance to that of Huia is less clear. Results for  $CoV_E$  suggest that Huia was intermediate to but not different from either of the Al-selected populations. However, emergence results indicate that the H-AIR-C1 population and its parent, Huia, were essentially identical in resistance to Al and that the major change was the increased sensitivity to Al of the H-AIS-C1 population. For either character, the H-ASR-C1 population was

similar to the H-AIR-C1 population and not different from Huia in Al sensitivity.

For either parent, we were able to select populations that diverged in Al resistance. Thus, both Brown Loam and Huia contain genetic variation for response to Al. For Huia, this finding is in agreement with the soil-based research, clearly indicating the presence of genetic variation for acid-soil resistance (Caradus et al., 1991; Caradus and Mackay, 1995; Caradus and Crush, 1996) although our Huia acid-soil-resistant population did not differ from Huia in seedling Al resistance. Changes in Al resistance in Brown Loam appears to have been bi-

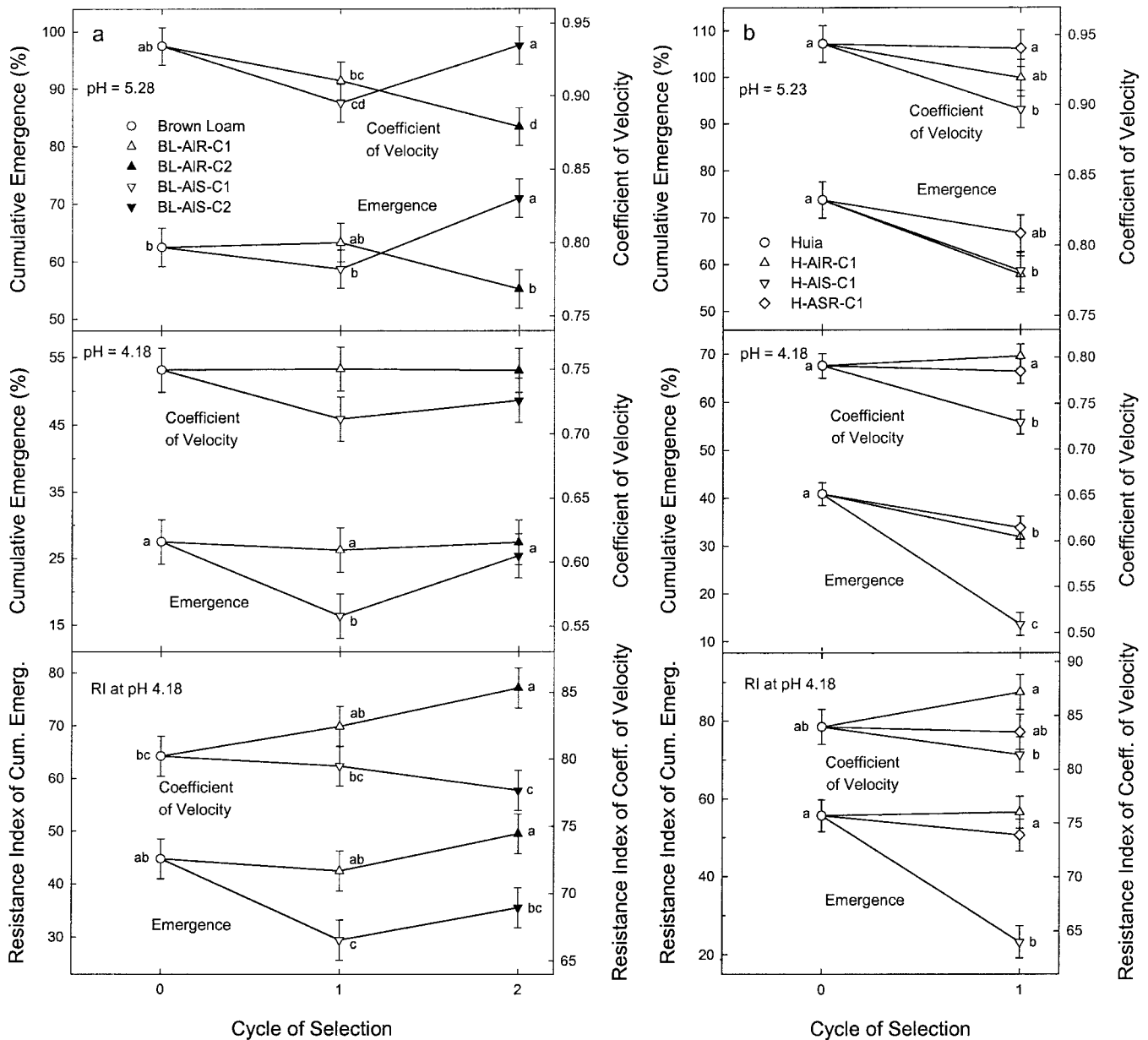


Fig. 2. (a) Cumulative root emergence and coefficient of velocity of root emergence (CoV<sub>E</sub>) of Brown Loam (BL), and the Al-resistant Cycles 1 and 2 (BL-AIR-C1, BL-AIR-C2) and susceptible Cycles 1 and 2 (BL-AIS-C1, BL-AIS-C2) white clover populations derived from it, at two levels of soil pH and resistance index, indicating Al resistance, of those characteristics at pH 4.18. (b) Cumulative root emergence and CoV<sub>E</sub> of Huia (H), and the Al-resistant Cycle 1 (H-AIR-C1) and susceptible Cycle 1 (H-AIS-C1) and acid-soil-resistant Cycle 1 (H-ASR-C1) white clover populations derived from it, at two levels of soil pH and resistance index, indicating Al resistance, of those characteristics at pH 4.18. Symbols within characters followed by the same letter are not significantly different at the 0.05 probability level. Vertical axis scales have been adjusted so that standard error bars for both characters are approximately equal in size.

directional, toward both greater Al resistance and susceptibility. Results for selection in Huia appear to have been more unidirectional, that is, more toward Al susceptibility than toward Al resistance. However, this conclusion must be viewed cautiously. The relationship between parent and offspring could have been impacted by the outstanding quality of the Huia seed and the excellent early seedling vigor of the resulting seedlings. These differences in seedling vigor would have been confounded with parent offspring comparisons. Where differences in Al resistance are relatively large, the sensitivity of primary root growth to toxic levels of Al can

overcome differences in seedling vigor and provide a useful assessment of Al sensitivity (Voigt and Mosjidis, 2002). Where differences in Al resistance are relatively small, a difference in seedling vigor could obscure the difference in Al resistance. In retrospect, we should have produced our own unselected Huia seed to use in these studies, thus avoiding the problem.

### Aluminum Resistance of Brown Loam Acid-Soil Populations

Root emergence curves (Fig. 3) for Brown Loam and its acid-soil populations at pH 5.23, were similar to

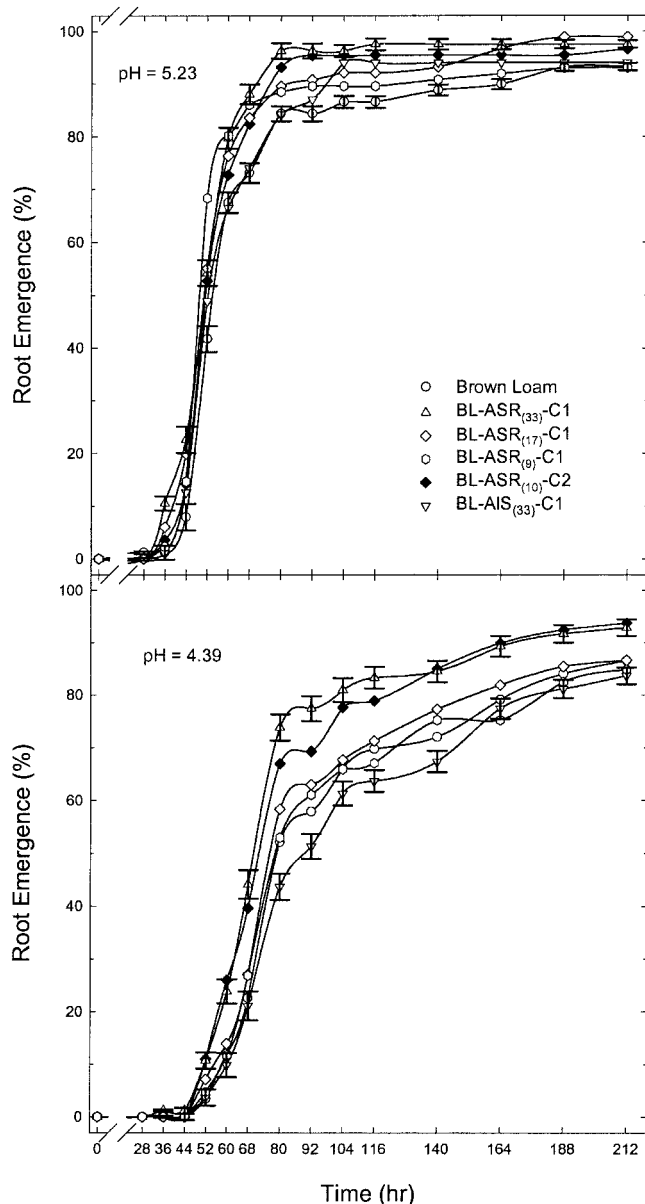


Fig. 3. Effect of soil pH on root emergence across time from soil into agar of Brown Loam (BL) and the acid-soil-resistant Cycles 1 and 2 (BL-ASR<sub>33</sub>-C1, BL-ASR<sub>17</sub>-C1, BL-ASR<sub>9</sub>-C1, and BL-ASR<sub>10</sub>-C2) and Al-susceptible Cycle 1 (BL-AIS<sub>33</sub>-C1) white clover populations derived from it. Standard error bars are shown for two populations but apply to all populations.

those at the high pH levels in the other experiments (Fig. 1), although there appeared to be less dispersion among populations in this experiment. At the lowest pH in this experiment (Fig. 3), pH 4.39, the delay in primary root emergence was clearly less and the level of emergence achieved more than that at the lower pH (4.18) of the first two experiments. Although dispersion among populations at pH 4.39 was less than that observed at the lower pH in the Huia experiment (Fig. 1b), the BL-ASR<sub>33</sub>-C1 and BL-ASR<sub>10</sub>-C2 populations were clearly superior in speed and total emergence compared with the other populations (Fig. 3).

Results from the third experiment (Fig. 4) are shown for T50E and CoV<sub>E</sub>. Note that the scales for these two

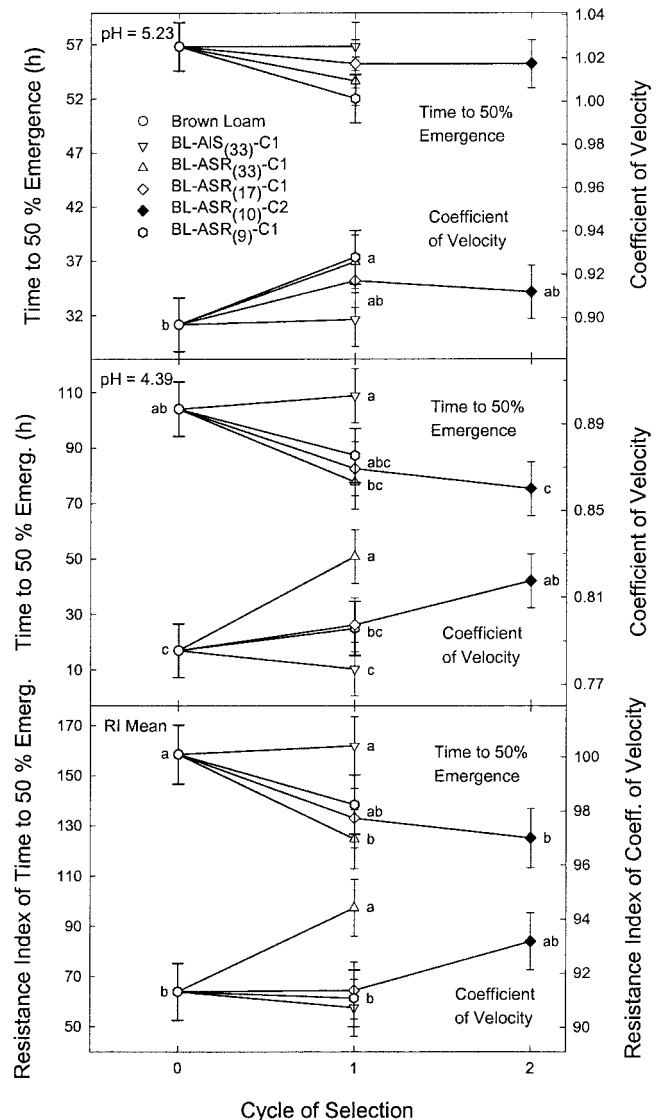


Fig. 4. Time to 50% root emergence and coefficient of velocity of root emergence of Brown Loam (BL) and the acid-soil-resistant Cycles 1 and 2 (BL-ASR<sub>33</sub>-C1, BL-ASR<sub>17</sub>-C1, BL-ASR<sub>9</sub>-C1, BL-ASR<sub>10</sub>-C2) and Al-susceptible Cycle 1 (BL-AIS<sub>33</sub>-C1) white clover populations derived from it, at two levels of soil pH and resistance index, indicating Al resistance, of those characteristics, mean of pH 4.39 and 4.64. Symbols within characters followed by the same letter are not significantly different at the 0.05 probability level. Vertical axis scales have been adjusted so that standard error bars for both characters are approximately equal in size.

characters run in opposite directions, that is a low T50E and a high CoV<sub>E</sub> indicate faster emergence. Few differences were detected at pH 5.23. In contrast, differences were observed at pH 4.39 for both characters ( $P < 0.05$ ). The BL-AIS<sub>33</sub>-C1 population was slower to emerge than the BL-ASR<sub>33</sub>-C1 population and the BL-ASR<sub>10</sub>-C2 population was faster to emerge than Brown Loam for both characters.

Results for resistance index indicate that the BL-ASR<sub>33</sub>-C1 population was more Al resistant than either the BL-AIS<sub>33</sub>-C1 population or Brown Loam, as measured by T50E or CoV<sub>E</sub> ( $P < 0.05$ ). The BL-ASR<sub>19</sub>-C1 and BL-ASR<sub>9</sub>-C1 populations were either intermediate



**Table 2. Number of leaves at Weeks 1, 4, and 9 of the acid-soil resistance experiment.**

		Leaves at Week		
Germplasm†	Soil pH <sub>w</sub>	1	4	9
		no.		
Brown Loam Synthetic No. 2	5.6	3.16a‡	6.41a	11.10a
	4.7	3.19a	6.30a	10.62a
	4.2	3.24a	6.10a	9.57b
	Mean	3.20B§	6.27B	10.43B
Grasslands Huia	5.6	3.58a	8.53a	14.35a
	4.7	3.74a	8.60a	13.92a
	4.2	3.75a	8.00b	11.94b
	Mean	3.69A	8.38A	13.40A

† Mean of parents and all populations derived from them.

‡ Values within columns and germplasm groups followed by different lowercase letters are significantly different at the 0.05 probability level by *t* test.

§ Germplasm group means within columns followed by different uppercase letters are significantly different at the 0.05 probability level by contrast statement.

or similar to the BL-ALS<sub>33</sub>-C1 population. The BL-ASR<sub>10</sub>-C2 population was also more Al resistant than Brown Loam, as measured by T50E ( $P < 0.05$ ), but was intermediate for CoV<sub>E</sub>. In an additional soil-on-agar experiment (data not shown), the BL-ASR<sub>10</sub>-C2 population was superior to Brown Loam in Al resistance for both emergence and CoV<sub>E</sub> ( $P < 0.05$ ). Our results indicate that both the BL-ALS<sub>33</sub>-C1 and BL-ASR<sub>10</sub>-C2 population had more seedling Al resistance than Brown Loam. Although there was some suggestion that more intensive selection for mature plant acid-soil resistance might have reduced seedling Al resistance (Fig. 4), when results from the additional experiment were considered also, we concluded that second-stage selection for mature plant acid-soil resistance probably did not adversely impact Al resistance at the seedling stage, although it certainly did not improve it.

### Acid-Soil Resistance of Experimental Populations

The week following transplanting of the pot experiment, mean number of fully expanded leaves per plant was 3.4. At that time there were no differences among pH treatments in number of leaves per seedling (Table 2). Huia and the populations derived from it always had more leaves than Brown Loam and the populations derived from it ( $P < 0.05$ ). Effects of the soil pH treatments appeared gradually and were detected for Huia germplasm in leaf counts made during the fourth week of the experiment ( $P < 0.05$ ). By Week 9 of the experiment, differences in leaf number were detected for both germplasm sources, but only between the pH 4.2 treatment and the higher pH levels ( $P < 0.05$ ). Results for leaf and for stolon weights were similar to those for root and top weight and will not be presented.

### Acid-Soil Resistance of Brown Loam Aluminum Populations

Top weight and root weight results for the Brown Loam Al selections were very similar at both pH 5.6 and 4.2 (Fig. 5a). Within cycles of selection there were no differences at Cycle 1, but the BL-ALS-C2 population yielded more tops and roots than the BL-AIR-C2 popu-

lation. At pH 5.6, but not at 4.2, the between cycle changes in weight of the Al-resistant populations were significant ( $P < 0.05$ ). Both the BL-ALS-C1 and BL-ALS-C2 populations had higher root and top yields than Brown Loam at either pH level ( $P < 0.05$ ).

For resistance index, there were no differences within cycles of selection and none of the selected populations differed from the Brown Loam parent. The differences in Al resistance of the primary roots of seedlings from the Al-resistant and susceptible populations (Fig. 2a) were not predictive of differences in acid-soil resistance at more mature stages of growth (Fig. 5a).

### Acid-Soil Resistance of Huia Populations

Results for the Huia-derived germplasm were somewhat different (Fig. 5b). Differences in top and root weight were not detected at pH 5.6 ( $P > 0.05$ ). At pH 4.2, however, all selected populations exceeded Huia in top weight and that of the H-ASR-C1 population was larger than that of H-AIR-C1 population ( $P < 0.05$ ). The trends for root weight were the same although fewer differences were detected.

Seedling Al-susceptible germplasm of both Huia and Brown Loam had higher root and top weights than their parents when those populations were grown to more mature stages in acid soil. We do not have an explanation for this observation.

Resistance index values for root weight indicate that the H-AIR-C1 population was more acid-soil resistant than Huia ( $P < 0.05$ ), but the H-ALS-C1 population was intermediate with only a slightly lower resistance index than the H-AIR-C1 population. For both root and top weight, the H-ASR-C1 population, derived through natural selection from a seeding on an acid field, had the largest resistance index. It was more acid-soil resistant than Huia ( $P < 0.05$ ).

Selection for seedling Al resistance produced inconsistent responses in acid-soil resistance of more mature plants. For Brown Loam, selection for seedling Al resistance did not alter mature-plant acid-soil resistance. Selection for Al resistance in Huia did results in increased acid-soil resistance, but the acid-soil resistance of the Al-susceptible population was almost as good. Selection for seedling Al resistance, based on primary root growth, does not appear to be an effective way to increase acid-soil resistance of more mature plants.

### Acid-Soil Resistance of Brown Loam Acid-Soil Populations

At pH 5.6, differences in top weight among Brown Loam acid-soil-resistant populations were minimal (Fig. 6). However, root weight differences were substantial and both root and top weight differences at pH 4.2 were large. Populations with high top and root weights at the lower pH were BL-ASR<sub>9</sub>-C1, BL-ASR<sub>10</sub>-C2, and BL-ALS<sub>33</sub>-C1.

For both root and top weight, resistance index values of the BL-ASR<sub>10</sub>-C2 population were larger than those for Brown Loam ( $P < 0.05$ ) or for the BL-ASR<sub>17</sub>-C1



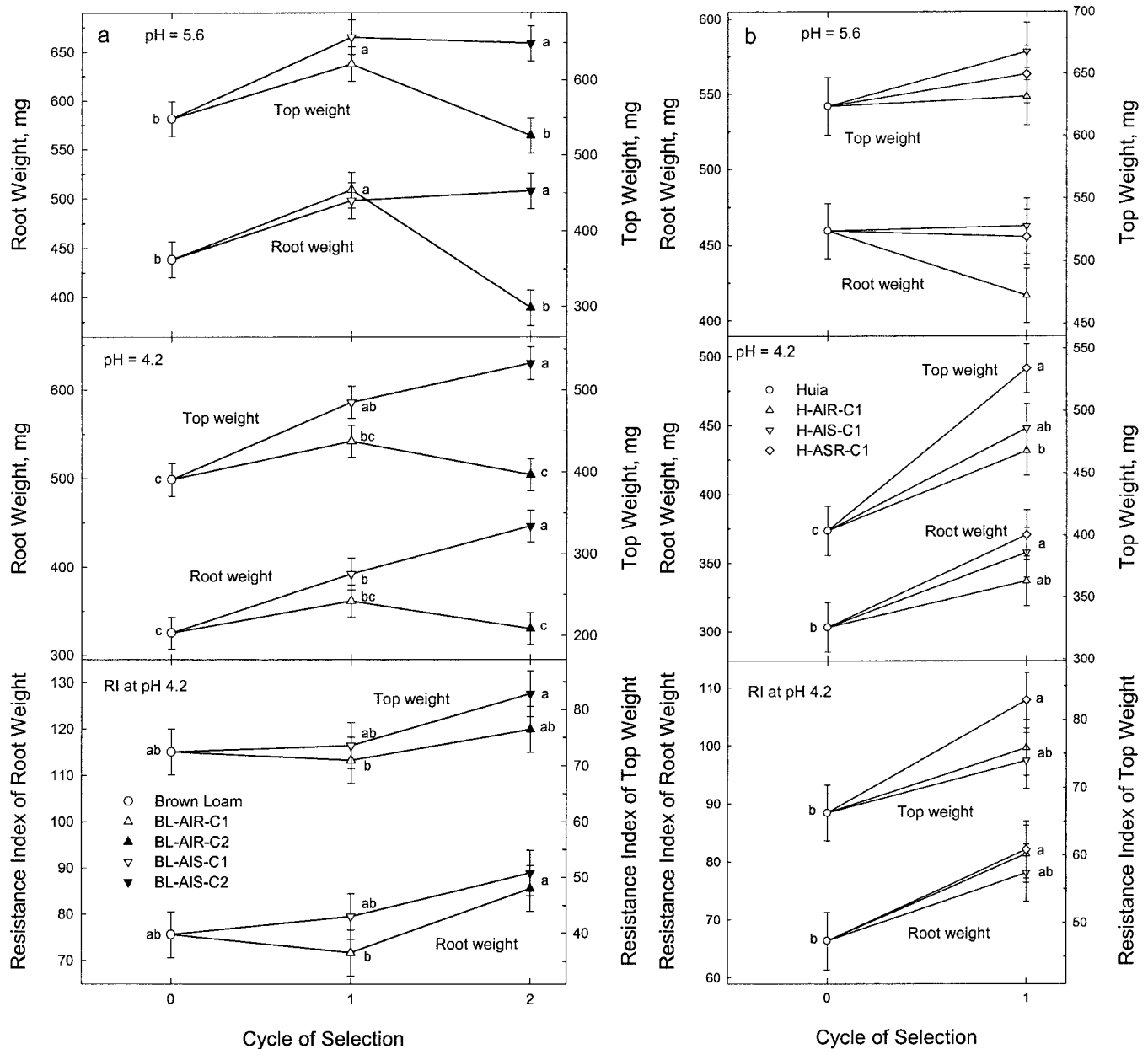


Fig. 5. (a) Top and root weight of Brown Loam (BL), and the Al-resistant Cycles 1 and 2 (BL-AIR-C1, BL-AIR-C2) and susceptible Cycles 1 and 2 (BL-AIS-C1, BL-AIS-C2) white clover populations derived from it, when grown at two levels of soil pH and resistance index, indicating acid-soil resistance, of those characteristics at pH 4.2. (b) Top and root weight of Huia (H), and the Al-resistant Cycle 1 (H-AIR-C1), the susceptible Cycle 1 (H-AIS-C1), and the acid-soil resistance Cycle 1 (H-ASR-C1) white clover populations derived from it when grown at two levels of soil pH and resistance index, indicating acid-soil resistance, of those characteristics at pH 4.2. Symbols within characters followed by the same letter are not significantly different at the 0.05 probability level. Vertical axis scales have been adjusted so that standard error bars for both characters are approximately equal in size.

population ( $P < 0.05$ ), the population from which BL-ASR<sub>10</sub>-C2 was derived. For top weight only, the most highly selected Cycle 1 population, BL-ASR<sub>9</sub>-C1, had a larger resistance index than Brown Loam ( $P < 0.05$ ) or the BL-ASR<sub>17</sub>-C1 population ( $P < 0.05$ ). The most intensely selected acid-soil-resistant populations had the highest acid-soil resistance.

Of the two populations that had shown the best Al resistance (Fig. 4), one, BL-ASR<sub>10</sub>-C2, also had better acid-soil resistance than Brown Loam (Fig. 6) while the second, BL-ASR<sub>33</sub>-C1, was no better than Brown Loam

( $P > 0.05$ ) in acid-soil resistance. Also, a population that was not especially Al resistant (Fig. 4), BL-ASR<sub>9</sub>-C1 was relatively acid-soil resistant. Within the acid-soil-resistant populations, there was not a close relationship between seedling Al resistance and mature-plant acid-soil resistance.

## CONCLUSIONS

The soil-on-agar technique can be used to develop populations of white clover that exhibit altered levels

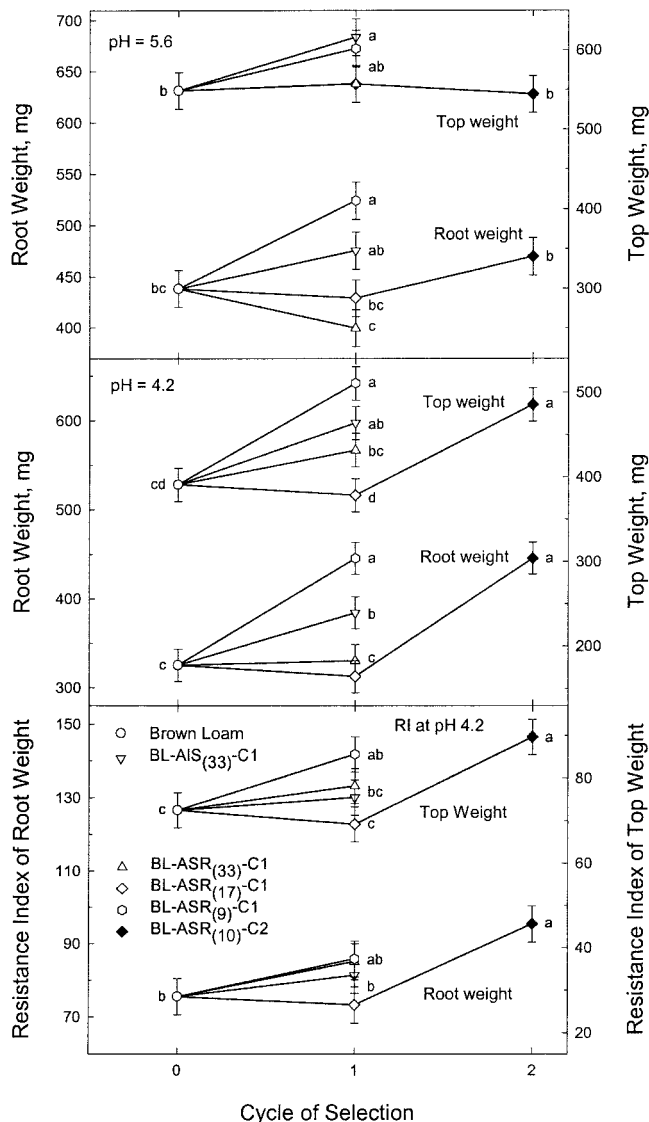


Fig. 6. Top and root weight of Brown Loam (BL), and the acid-soil-resistant Cycles 1 and 2 (BL-ASR<sub>33</sub>-C1, BL-ASR<sub>17</sub>-C1, BL-ASR<sub>9</sub>-C1, BL-ASR<sub>10</sub>-C2) and Al-susceptible Cycle 1 (BL-AIS<sub>33</sub>-C1) white clover populations derived from it, when grown at two levels of soil pH and resistance index, indicating acid-soil resistance, for those characteristics at pH 4.2. Symbols within characters followed by the same letter are not significantly different at the 0.05 probability level. Vertical axis scales have been adjusted so that standard error bars for both characters are approximately equal in size.

of Al resistance as expressed in the seedling stage by primary root growth. We have not studied primary root growth of our selections in solution culture. Thus, although we believe it unlikely, we cannot exclude the possibility that our soil-based estimates of Al resistance would be somewhat different from those obtained from classical solution culture techniques.

Although selection for seedling Al resistance and susceptibility in white clover was successful, seedling Al resistance was not closely related to mature plant acid-soil resistance. We do not know if this difference was because of a lack of correspondence between stages of growth, seedling Al resistance based on primary root growth vs. Al resistance at the more mature stages where

acid-soil resistance was measured, or because Al resistance was only one of several factors necessary for developing acid-soil-resistant white clover.

Studies of variation in Al resistance of white clover, as determined by classical solution culture techniques, indicate the presence of wide variation among white clover cultivars (Wheeler and Dodd, 1995). Yet soil-based studies show that variation among white clover cultivars was unimportant and that none were more acid-soil resistant than Huia (Caradus et al., 1987; Mackay et al., 1990). This difference in results suggests that at least a part of the reason for the failure of our white clover Al-resistant and susceptible selections to be also acid-soil resistant and susceptible is that factors in addition to Al resistance are required for white clover plants to be acid-soil resistant.

Brauer et al. (2002) described the growth of primary and secondary roots of Huia white clover in pH 4.8 and 5.3 Gilpin soil. Responses of primary root and of secondary determinate root lengths across time were linear, regardless of pH, although rates of growth differed. Secondary indeterminate root length, however, increased exponentially at the higher pH but only slightly at the lower pH. Similarly, in soybean [*Glycine max* (L.) Merr.], lateral roots are more sensitive to Al than the primary root (Sanzonowicz et al., 1998; Ferrufino et al., 2000; Silva et al., 2001). It is possible that the failure of primary root Al-resistant germplasm to be also acid-soil resistant at later stages of growth is that selection based on the primary root did not impact equally types of roots found in more mature plants or allow for acid-soil resistance that might develop gradually across time (Bushamuka and Zobel, 1998).

Both natural selection for acid-soil resistance in a field environment, from a base of Huia white clover, and artificial selection for acid-soil resistance in controlled environments, from a base of Brown Loam, were successful in increasing the acid-soil resistance of the base population, as long as the intensity of selection was sufficiently high. Thus, large-leaved white clover, like medium-small leaved white clover, contains genetic variation for acid-soil resistance. Also, the population derived by natural selection was selected in one soil (Gilpin) and evaluated in a second (Porters). Thus, acid-soil resistance was not soil specific, although results for additional soils might vary (Villagarcia et al., 2001).

The potential usefulness of our acid-soil-resistant germplasm is unknown. Caradus et al. (2001) transplanted white clover accessions selected for acid-soil resistance and susceptibility into two acid soils, pH 4.6 and 4.8, containing high levels of Al. They reported that "differences were not statistically significant because of the wide variation among replicates." The presence and condition of nodules was not observed. Our experience with white clover transplanted into an acid soil indicates that nodulation is not always maintained. We transplanted inoculated white clover into a Gilpin soil, pH of 4.2, (Voigt and Morris, 1996, unpublished data). When propagules of the most vigorous plants were examined 2 yr later, most propagules were devoid of nodules. The few nodules found were very small and probably not effective.

tive. Thus, at least a part of the decline in vigor that we had observed during those 2 yr, as well as that reported by Caradus et al. (2001) across 4 yr, could have been caused by the failure of white clover plants to remain effectively nodulated. Improved rhizobia, with increased ability to nodulate white clover in acid soils, may be necessary before the potential of acid-soil-resistant white clover germplasm can be fully realized.

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